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MARK F. STINSKI[1] AND JACK GRUBER[2]
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Medical Sciences Laboratory, Fort Detrick, Frederick, Md.

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MARK F. STINSKI[1] AND JACK GRUBER[2]
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Medical Sciences Laboratory, Fort Detrick, Frederick, Maryland 21701

Untreated suspensions of group B arboviruses Japanese B encephalitis (1) and dengue-2 (2) possess a noninfectious slow-sedimenting component (NSCF), that fixes complement in the presence of appropriate antisera. In contrast, untreated suspensions of Semliki Forest virus, a group A arbovirus, do not possess a similar component (3). A NSCF component was isolated from Sindbis virus, a group A arbovirus, only after treatment with sodium deoxycholate (4) or Tween–ether (5). Since group A and group B arboviruses are differentiated on the basis of their serological antigens, further knowledge of the biophysical characteristics of these antigens would be desirable.

To isolate and characterize the physical properties of group B arbovirus NSCF components, suspensions of West Nile (WN), Ilheus (ILH), and Rio Bravo (RB) viruses were analyzed by sucrose density gradient centrifugation. For comparison, a representative group A arbovirus, Venezuelan equine encephalitis (VEE) was also analyzed.

Materials and Methods. Cell culture. Chicken embryonic cell (CEC) culture was prepared from 10-day-old chicken embryos and grown in a medium consisting of 0.5% lactalbumin hydrolysate; 0.1% yeast extract; 10% calf serum; 100 units of penicillin/ml; 100 μg of streptomycin/ml; and 0.14% sodium bicarbonate in Hanks' balanced salt solution.

Mouse L cells were grown in Nagle's CSA-1 medium (6) supplemented with calf serum and antibiotics as described above and with 0.1 m*M* L-glutamine.

Virus preparations. The Trinidad strain of VEE virus, originally isolated in guinea pigs from a donkey brain (7), was obtained through the courtesy of our colleague William P. Allen. The virus was grown in CEC from a seed prepared after a second suckling mouse brain passage. Virus-CEC harvests were centrifuged at 12,000*g* for 30 min and the clarified supernatant fluid was centrifuged at 78,000*g* for 3 hr. The resulting sediment was resuspended to 1/100 the original volume in phosphate-buffered saline, pH 9.0, containing 0.1% bovine serum albumin. The preparation contained 10.5 $\log_{10}$ mouse intracerebral 50% lethal doses (MI CLD_{50})/ml.

Original isolations of viruses WN (strain Eg 101), ILH (strain Detrick), and RB (strain HA 119) were used after five or six suckling mouse brain passages. Twenty percent infected suckling mouse brain suspensions in 4% bovine albumin–borate saline, pH 9.0, were centrifuged at low speed (12,000*g*, 1 hr, 5°) and the clarified supernatant fluids were collected. These preparations contained approximately 9.5 $\log_{10}$ $MICLD_{50}$/ml.

Antiserum and ascitic fluids. Hyperimmune anti-VEE virus serum, obtained from W. P. Allen, was prepared in horses by initial inoculation of attenuated virus following by five inoculations of virulent virus. Hyperimmune ascitic fluids for viruses WN, ILH, and RB were prepared in mice (8) with live virus inoculations. An additional virus inoculation was made after intraperitoneal implantation of sarcoma cells.

Infectivity assays. Infectivity of VEE, WN, ILH, and RB viruses were assayed by titration of lethality following intracerebral

[1] Present address: CPT, 392-38-9630, HHB 7th Inf. Div. Arty., APO, San Francisco, California, 96251.

[2] Present address: Viral Biology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

inoculation of 10- to 12-g weanling mice. Viruses WN, ILH, and RB also were assayed for infectivity by plaque titration using confluent 1- to 2-day-old mouse L cell monolayers. Virus samples were adsorbed to monolayer cultures for 1 hr at 37°, and then medium containing 1% Noble agar was added. The monolayers were incubated at 34° for 4 more days and then stained with medium containing agar and 0.1% neutral red. Plaques were counted after 1 day's incubation at 34°. For WN, ILH, and RB viruses, the plaque and mouse infectivity assay systems gave similar results.

Antigenicity assays. Hemagglutinating (HA) and complement-fixing (CF) activities were measured by microtiter techniques (9, 10). Hemagglutinating activity was titrated with goose erythrocytes. The reaction mixture was buffered at pH 5.8 for VEE virus, pH 6.2 for ILH and RB viruses, and pH 6.4 for WN virus. In the CF test, the highest dilution of antigen with 30% hemolysis or less was taken as the end point

Density gradient centrifugation. Virus suspensions of 0.5 and 3 ml were analyzed by rate zonal centrifugation in 5- and 30-ml linear sucrose gradients, respectively. The gradients were constructed of 5 and 30% or 5 and 40% (w/w) ribonuclease (RNase)-free sucrose (Mann Research Laboratory, New York) dissolved in borate-buffered saline solution, pH 9.0. Human hemoglobin (Pentex Inc., Kankakee, Ill.), 0.05 mg, was used as a sedimentation marker. After centrifugation at 50,000*g* for 2 or 3 hr at 5°, 0.15 or 1.5 ml fractions were collected dropwise through a hole punctured in the tube bottom and analyzed for hemoglobin absorbence at 412 mμ, infectivity, HA, and CF activity. Approximate sedimentation coefficients for slow-sedimenting components were calculated (11) with the assumed value of 4.2 S for human hemoglobin (12).

To determine the buoyant densities of the HA and CF components in virus suspensions of WN, ILH, and RB, 0.5-ml samples were layered onto 5-ml gradients of 15 and 55% (w/w) RNase-free sucrose prepared as described above. The preparations were centrifuged at 73,500*g* for 20 hr at 15°. The fraction were collected and analyzed as described above. Densities of every other fraction were determined at 20° with an Abbe-3L Bausch and Lomb refractometer. From these values a straight line was calculated by regression analysis.

Results. Analysis for infectivity, HA, and CF activity. All HA and CF activity of group A arbovirus VEE was coincident with virus infectivity, which sedimented as a single entity in sucrose gradients (Fig. 1). In contrast, evident HA components with less than 1% of the total recovered infectivity were detected in preparations of WN and ILH viruses (Fig. 2a,b). A similar noninfectious HA anti-

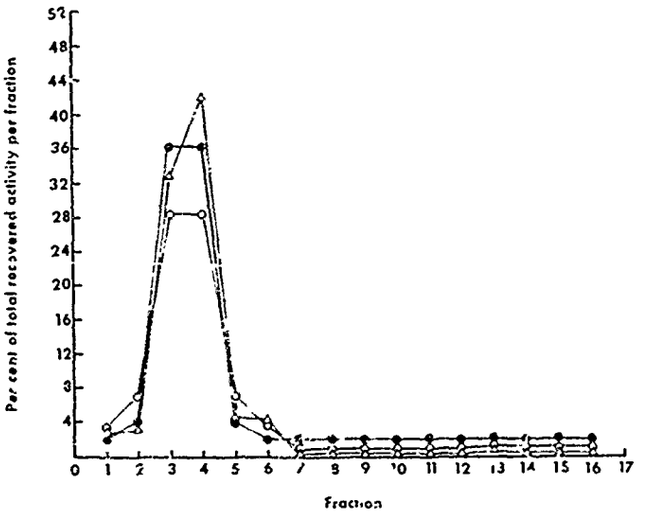

FIG. 1. Rate zonal centrifugation of VEE virus in a sucrose gradient: (△), infectivity; (○), hemagglutination; (●), complement fixation.

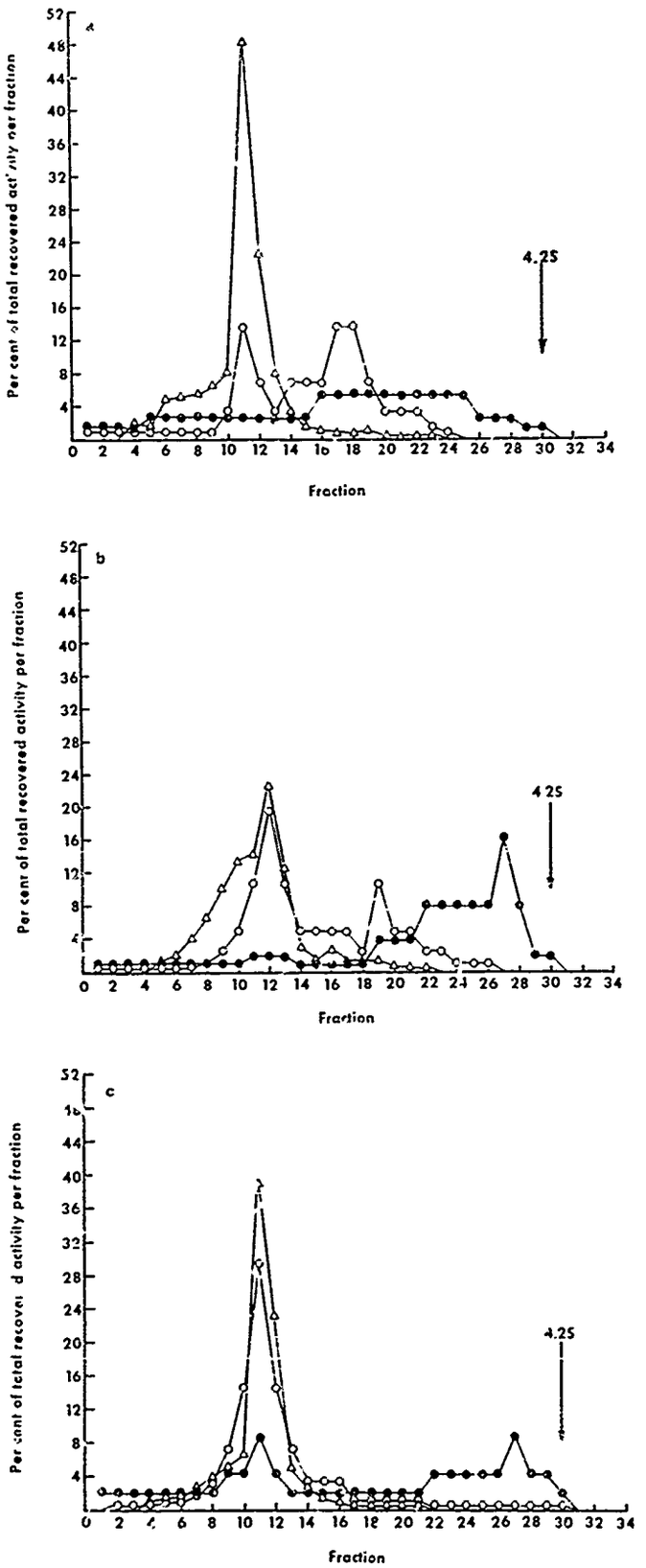

FIG. 2. Rate zonal centrifugation of WN (a), ILH (b), and RB (c) viruses in sucrose gradients: (△), infectivity; (○), hemagglutination; (◑), complement fixation.

TABLE I. Hemagglutination-Inhibition and Complement-Fixation Tests for WN, ILH, and RB Viruses and Their Isolated Antigenic Components.

Antigenic components	Immune ascitic fluids					
	WN		ILH		RB	
	HI titer	CF titer	HI titer	CF titer	HI titer	CF titer
WN original virus preparation	*512*[a]	*640*[b]	256	80	256	<5
WN infectious HA	*1024*	*10*	512	5	512	<5
WN noninfectious HA	*1024*	*10*	512	5	512	<5
WN noninfectious slow-sedimenting complement-fixing (NSCF)	*ND*[c]	*40*	ND	<5	ND	<5
ILH original virus preparation	256	40	*1024*	*1280*	512	5
ILH infectious HA	128	5	*1024*	*10*	256	<5
ILH noninfectious HA	128	5	*1024*	*10*	256	5
ILH NSCF	ND	<5	*ND*	*80*	ND	<5
RB original virus preparation	512	<5	512	<5	*1024*	*320*
RB infectious HA	128	<5	256	5	*1024*	*5*
RB NSCF	ND	<5	ND	<5	*ND*	*20*

[a] Reciprocal of the dilution of immune ascitic fluid showing complete inhibition of 10 HA units. Italicized numbers indicate homologous reactions.

[b] Reciprocal of the dilution of antigen with 30% or less hemolysis.

[c] HI not done due to lack of a hemagglutinin.

gen was not detected in RB virus preparations (Fig. 2c). A noninfectious (0.002% of total recovered infectivity) slow-sedimenting complement-fixing (NSCF) component with little or no HA activity was detected in all the group B arbovirus preparations. This component sedimented faster than 4.2 S human hemoglobin (Fig. 2). A sedimentation coefficient of approximately 6S was calculated for the NSCF component in ILH and RB virus suspensions. A similar calculation was not possible for the WN NSCF component because no sharply defined peak of CF activity was observed.

Buoyant densities of HA and CF antigens. The infectious HA components of WN, ILH, and RB viruses all had buoyant densities of approximately 1.19 g/cc (Fig. 3a,b,c). The noninfectious HA components of viruses WN and ILH had buoyant densities of approximately 1.16 g/cc (Fig. 3a,b). Only 2 to 7% of the total recovered CF activity was present at densities of 1.19 and 1.16 g/cc. However, maximum recovery of CF activity, 14 to 17%, was at buoyant densities of 1.10 to 1.12 g/cc (Fig. 3a,b,c).

Serologic relatedness of viruses and components. Hemagglutination-inhibition (HI) and complement-fixation (CF) tests with homologous and heterologous immune ascitic fluids were conducted with WN, ILH, and RB viruses and their isolated antigenic components. All viral infectious and noninfectious hemagglutinins (HA) reacted with homologous immune ascitic fluids (Table I). Cross-reaction of WN, ILH, and RB viruses and their isolated HA components with heterologous ascitic fluids was observed by the HI test. Antigenic relatedness of WN and ILH viruses was demonstrable by the CF test. However, the same test failed to detect a significant antigenic relationship of RB virus to WN and ILH viruses (Table I). All NSCF components reacted with homologous immune ascitic fluids but no cross-reactions were detected with heterologous ascitic fluids (Table I).

Discussion. Noninfectious slow-sedimenting complement-fixing (NSCF) components had been detected in untreated suspensions of Japanese B encephalitis (1) and dengue-2 (2) viruses, group B arboviruses. The present data indicate that NSCF components are also present in untreated suspensions

of the group B arboviruses WN, ILH, and RB. These antigens were present in virus suspensions prepared from infected suckling mouse brain (1, 2) or cultured cells (2). Similar NSCF components were not found in untreated suspensions of the group A arbovi-

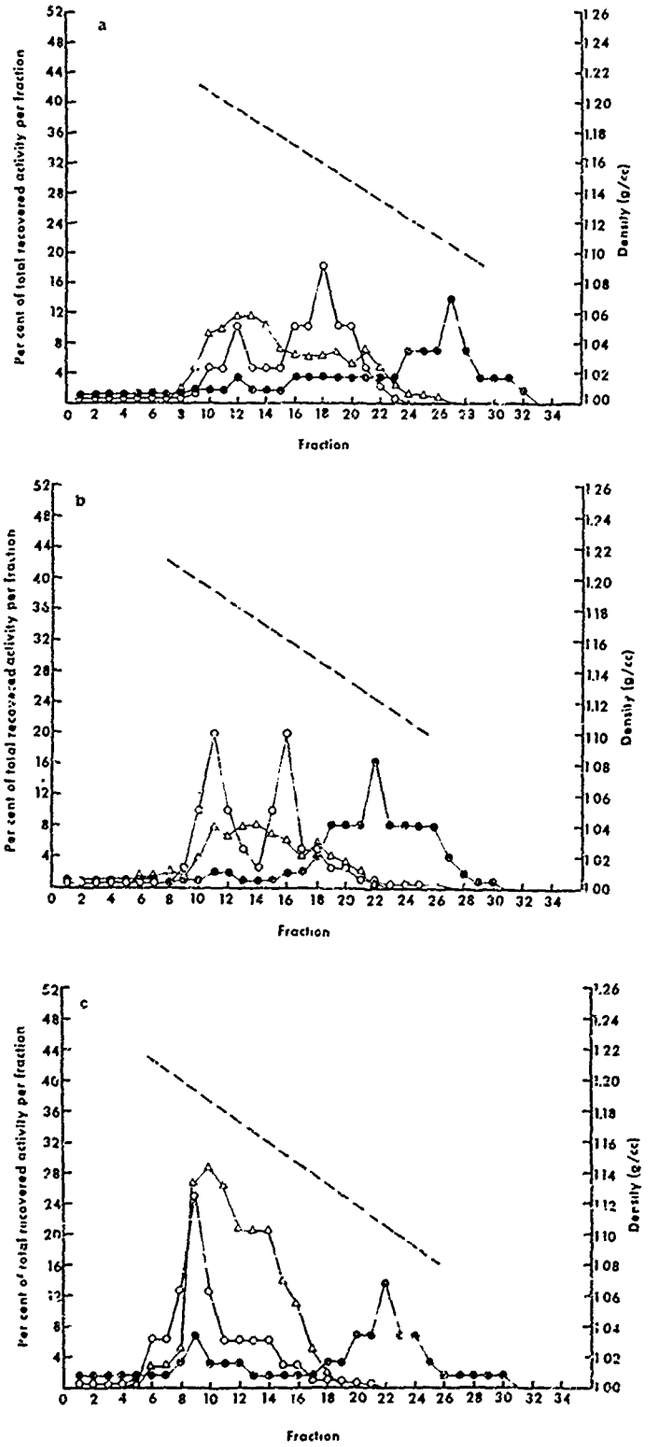

FIG. 3. Isopycnic centrifugation of WN (a), ILH (b), and RB (c) viruses in sucrose gradients: (△), infectivity; (○), hemagglutination; (●), complement fixation; (- - - -), g/cc.

ruses Semliki Forest (3), Sindbis (5), and, in the present study, Venezuelan equine encephalitis. Only after Sindbis virus was disrupted by treatment with reagents such as sodium deoxycholate (4) or Tween–ether (5) were NSCF components detected. It is conceivable that the failure to detect VEE NSCF components is a function of the horse antiserum used; this possibility seems remote. Evidently the presence of NSCF components in untreated viral suspensions is characteristic of group B but not of group A arboviruses.

The NSCF components in untreated suspensions may be viral proteins that were not assembled into the virion, or may be proteins from fragmented viruses. If the former, these viral proteins could be structural or nonstructural in nature. Fragmentation of group B viruses may be induced by ultracentrifugation. The NSCF components of Japanese B encephalitis (1) and dengue-2 (2) viruses were detected in preparations pelleted by ultracentrifugation. However, the NSCF components of WN, ILH, and RB viruses detected in this investigation and those of dengue-2 virus (2) were present in nonpelleted preparations.

The chemical nature of the density gradient affects virus stability. Cesium chloride gradients were reported to disrupt Eastern equine encephalitis virus (13) and dengue-2 virus (2), but sucrose gradients had no disruptive effect even after repetitive centrifugation. Consequently, sucrose gradients were used in this study and there was no evidence that disruption of the viruses occurred during gradient centrifugation.

Virus samples were frozen and stored at −70° prior to gradient analysis. It is possible that this manipulation induced some fragmentation of the group B viruses. However, NSCF components were not detected after similar manipulations with VEE virus. The present data do not eliminate the possibility that the difference between group A and group B viruses reflects a difference in stability at some stage of the experimental procedure. However, it seems more probable that the NSCF components represent unassembled viral antigens that are characteristically present in infected cells.

Knowledge of NSCF components may prove useful in developing new methods of viral immunization. Treatment of Sindbis (4) and vesicular stomatitis (14, 15) viruses with sodium deoxycholate or Tween–ether disrupted the viruses, releasing nucleocapsids and envelopes separable by sucrose gradient centrifugation. The Sindbis virus envelope sedimented slowly, had CF activity, and absorbed neutralizing antibodies. Vesicular stomatitis virus (VSV) envelope was noninfectious, immunogenic in guinea pigs, and the sedimentation coefficient (6S) was similar to the NSCF components of ILH and RB viruses. Untreated suspensions of VSV contained NSCF components that were also immunogenic in guinea pigs (16). It is possible that the NSCF components of the group B arboviruses characterized in this study may prove to be immunogenic.

Summary. Sucrose gradient analysis of Venezuelan equine encephalitis virus, a group A arbovirus, indicated that hemagglutinating (HA) and complement-fixing (CF) activities were coincident with virus infectivity. In contrast, West Nile, Ilheus, and Rio Bravo viruses of group B were fractionated into infectious and noninfectious components. The infectious component sedimented as a single entity, possessed HA and CF activity, and had a buoyant density of 1.19 g/cc. West Nile and Ilheus virus suspensions contained a slower sedimenting noninfectious HA and CF component with a buoyant density of 1.16 g/cc. Rio Bravo virus suspensions did not contain a similar component. A very slow-sedimenting noninfectious CF component (6 S, 1.10–1.12 g/cc) was detected for each of the group B viruses.

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